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Chemokine Expression in Murine RPE/Choroid in Response to Systemic Viral Infection and Elevated Levels of Circulating Interferon- γ

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PURPOSE. To examine how circulating immune mediators in vivo may affect gene and protein expression at the RPE/choroid interface.

METHODS. Young mice were systemically infected with lymphocytic choriomeningitis virus (LCMV) or continuously infused with IFN- γ . RPE/choroid was isolated and analyzed with whole-transcriptome gene expression microarrays. Selected gene expression findings were validated at the protein level.

RESULTS. Both the systemic immune activation from virus infection and the sterile systemically increased level of IFN- γ resulted in increased expression of chemokine ligands, chemokine receptors, and early complement components in isolates of RPE/choroid. These findings were largely absent from LCMV-infected mice deficient in either the interferon α/β receptor or IFN- γ .

CONCLUSIONS. Together, these findings demonstrate that acute systemic immune activation results in a local response at the RPE/choroid interface that may include chemokine-dependent recruitment of inflammatory cells and engagement of the complement system. This may represent a link between the systemic low-grade inflammation and the retinal pathology observed in several multifactorial entities such as aging, AMD, and diabetes.

Keywords: chemokine, complement, interferon, LCMV, retina, RPE

Several entities affecting the retinal function such as diabetes,¹ AMD,^{2–4} and aging⁵ have all been associated with systemic low-grade inflammation. Conversely, diseases characterized by systemic inflammation have been associated with ocular diseases. Acquired immunodeficiency syndrome and myeloproliferative neoplasms have been associated with a higher incidence of AMD,^{6,7} and diverse inflammatory diseases including inflammatory bowel disease and arthritis have been associated with an increased risk of posterior uveitis.^{8,9} However, the mechanism by which systemic inflammation is connected to ocular disease has not been agreed on.

The immune status of the chorioretinal interface is to a large extent governed by the RPE. Through the constitutive release of immunomodulatory molecules, the RPE can suppress the activation of T cells and promote the generation of regulatory T cells.^{10,11} Upon various stimuli in vitro, the RPE induces and increases the expression of cytokines,¹² chemokines,¹³ and early complement components.¹⁴ These stimuli include the inflammatory mediators lipopolysaccharide (LPS), IL-1 β , TNF- α , and/or IFN- γ . Less is known about possible immunomodulatory functions of the choroid. However, recently we have demonstrated that native choroidal melanocytes may harbor similar functionality including secretion of chemokines and suppression of T cell activation.¹⁵

Systemically increased levels of IFN- γ from systemic virus infection in rodents has been shown to induce a transient subretinal accumulation of retinal microglia,¹⁶ which indicates that systemic inflammation contributes to the immune status of the chorioretinal interface. The transcriptional response of the choroidal/RPE interface to systemic inflammation has, however, not been explored before.

Furthermore, while it has been demonstrated that aging activates the RPE/choroid in rodents to upregulate the expression of complement, chemokines and leukocyte adhesion molecules,^{17–19} it has not been determined to what extent this is due to age-related systemic low-grade inflammation or if it reflects ocular aging processes such as accumulation of lipofuscin or protein modifications from free-radical induced oxidative processes.

The blood supply to the RPE and outer neuroretina derives from the outer choroid, which is among the most highly perfused tissues in the body.²⁰ Therefore, we hypothesized that RPE/choroidal-exposure to circulating immune mediators may affect retinal immune homeostasis. To examine this, we measured transcriptional changes in the RPE/choroid in response to systemic viral infection and cytokine infusion, and identified IFN- γ as a central and sufficient mediator of RPE/choroid immune activation.



METHODS

Animals

WT C57BL/6 mice were obtained from Taconic Farms (Ry, Denmark). Interferon- α/β receptor (IFNAR)^{-/-} and IFN- γ ^{-/-} C57BL/6 mice were the progeny of mice originally obtained from The Jackson Laboratory (Bar Harbor, ME, USA). All mice were housed under specific pathogen-free conditions and used for experiments at the age of 8 to 12 weeks; all mice from outside sources were allowed to acclimatize to the local environment for at least 1 week before entering into experiments. Unless stated otherwise, all experimental groups contained four mice.

Induction and Measurement of Systemic Immune Activation

Systemic immune activation was induced by infection with lymphocytic choriomeningitis virus (LCMV) of the Armstrong strain or mimicked by continuous infusion with recombinant murine IFN- γ (Peprotech, Rocky Hill, NJ, USA). For virus infection, 10⁴ plaque-forming units in 0.3 mL PBS were injected into a tail vein. Intravenous inoculation of the low-invasive Armstrong strain of LCMV is known to infect the lymphoid organs, particularly the spleen, and elicit a potent antiviral CD8 T cell response with a substantial spill-over of proinflammatory cytokines into the general circulation.²¹ IFN- γ was delivered as previously described.²² Briefly, 7-day osmotic pumps (Durect, Cupertino, CA, USA) were filled with 50 μ g IFN- γ and placed in a subcutaneous pocket in Avertin-anesthetized mice. Negative controls included untreated mice and mice receiving PBS-filled pumps. At the end of experiments, at time points indicated, mice were bled into heparinized tubes, killed by cervical dislocation, and ocular tissue and spleens were removed. To verify immune activation, blood and spleens were analyzed. Blood was centrifuged at 1000g for 15 minutes and the plasma obtained was assayed for levels of IFN- α and IFN- γ using bead-based multiplex assay according to the manufacturer's instructions (eBioscience, Bender Medsystems, Vienna, Austria). Spleens were forced through a mesh, washed twice in RPMI + 10% FCS, and 10⁶ cells were transferred per well to a 96-well plate in PFEA buffer (PBS with 2% FCS, 2 mM EDTA and 0.01% azide). Cells were then stained in the dark for 30 minutes/4°C with a mixture of anti-mCD4-APC, anti-mCD8-APC-Cy7, anti-mCD44-PE-Cy7, and anti-mCD62L-PE (all from Biolegend, San Diego, CA, USA). After washing in PFEA buffer, acquisition of data was performed on an LSR-II instrument (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed using commercial software (FlowJo, version 7.6.5 for Windows; Tree Star, Inc., Ashland, OR, USA). Lymphocytes were gated from single cells based on light scatter profiles in the forward and side scatter detectors. Gates for CD4⁺ and CD8⁺ within the CD3⁺ T cell subset were then analyzed for frequencies of effector memory (CD44⁺ CD62L⁻) subsets.

Harvest of Ocular Tissues

Ocular tissues were removed immediately following cervical dislocation as previously described.¹⁹ Briefly, for the isolation of RNA, eyes were hemidissected, the neuroretinas were removed and the remaining RPE/choroid in the interior of the eyecups was scraped off with a 25G needle and collected in RNA lysis buffer containing reducing agent. RNA was isolated using spin columns (Nucleospin RNA XS; Macherey-Nagel, Düren, Germany). RPE/choroid from right and left eyes were pooled; no tissues from different mice were pooled. For the isolation of protein, tissue fractions of RPE/choroid were homogenized in

PBS containing a protease inhibitor cocktail (Sigma-Aldrich Corp., Copenhagen, Denmark) and 0.05% Triton X-100 as previously described.²³ Homogenates were centrifuged at 3000g for 30 minutes and the supernatants were stored at -80°C until protein analysis.

Microarrays

At a microarray core-facility (Center for Genomic Medicine, Copenhagen University Hospital, Copenhagen, Denmark), RNA was processed for murine whole-transcriptome microarrays (Mouse Gene 1.0 ST Array GeneChips; Affymetrix, Santa Clara, CA, USA) according to the manufacturer's protocol (Affymetrix GeneChip; Affymetrix). Probe level data was processed using an robust multichip average algorithm for adjustment of background and normalization (Expression Console 1.1; Affymetrix). Unannotated probe sets and probe sets with average expression levels below the median in two groups were removed from further analysis. Differentially expressed genes were detected with a false discovery rate (FDR) < 0.001 and a fold change >1.5 using the significance analysis of microarrays (SAM version 4.0; Stanford University, Stanford, CA, USA).²⁴ Gene expression values were normalized across samples using the formula ([expression value] - mean[column]/SD[column]) and displayed as heatmaps generated in the multiple experiment viewer (MeV version 4.8; Dana-Farber Cancer Institute, Boston, MA, USA).²⁵ Functional annotation of the differential gene expression was carried out using a classification tool (DAVID Bioinformatics Resources 6.7; National Institute of Allergy and Infectious Diseases, NIH, david.abcc.ncifcrf.gov).²⁶ Raw data CEL files have been deposited in the NCBI Gene Expression Omnibus under accession number GSE73519.

Chemokine Protein Measurements in Tissue Lysates and Plasma

Tissue homogenates and plasma samples were assayed for the concentration of murine CCL2, CCL3, CCL5, and CXCL9 using a multiplex cytometric bead-based assay (BD Biosciences). Chemokine concentrations were normalized to protein content according to a standard bicinchoninic acid (BCA) protein quantification assay.

Statistics

We used 1-way ANOVA with Dunnett's Multiple Comparison Test for analysis of cytokine and chemokine protein data and flow cytometry data (GraphPad Prism version 4.03; GraphPad, La Jolla, CA, USA). *P* values below or equal to 0.05 were considered statistically significant. Statistical analysis of the microarray gene expression data was carried out using the significance analysis of microarrays as described in the microarray section.

Study Approval

Experiments were approved by the Danish Animal Experiments Inspectorate and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

RESULTS

Systemic LCMV Infection Results in T Cell Activation and Increased Levels of Interferons in the Circulation

As a model of systemic immune activation, mice were systemically infected with LCMV. We evaluated the virus-

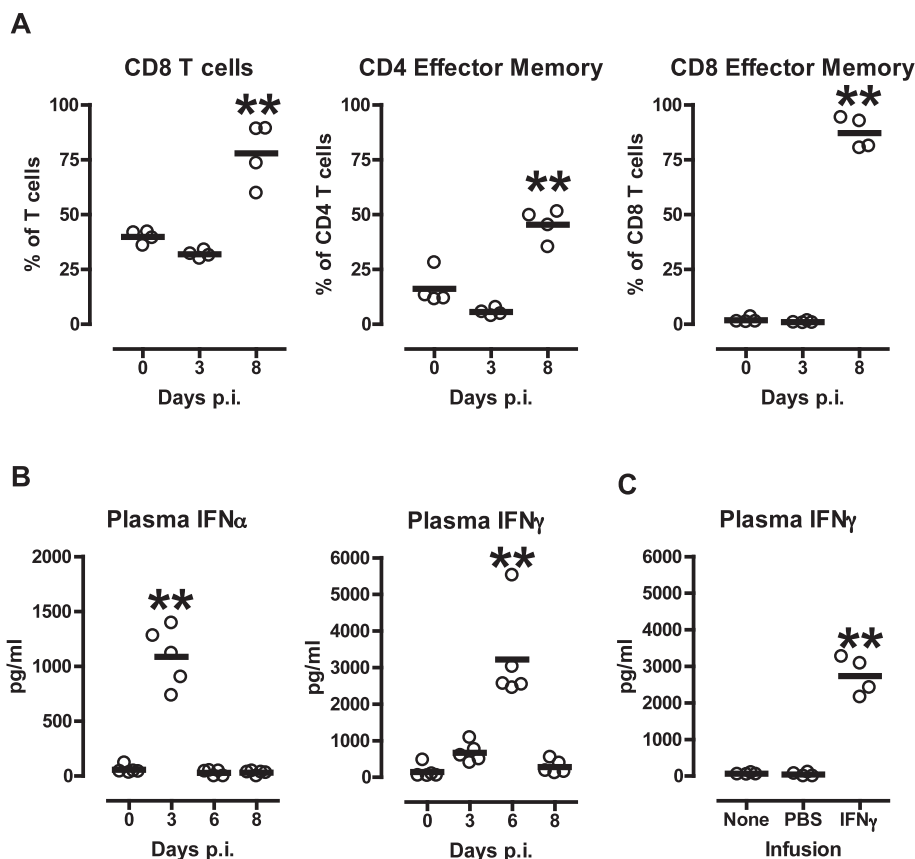


FIGURE 1. Systemic immune activation by LCMV infection. **(A)** The splenic CD8 T cell compartment expanded day 8 PI. This included expansion of effector/effector memory (CD44⁺ CD62L⁺) CD4 and CD8 subsets. **(B)** The plasma concentrations of both IFN- α and IFN- γ were increased at day 3. At day 6 PI IFN- α returned to the basal level while the IFN- γ level was increased further. At day 8 PI, plasma IFN- γ level had also returned to the basal level. **(C)** Plasma concentration of IFN- γ in mice 5 days after implantation of IFN- γ pumps corresponding to the time point for microarray analysis; 1-way ANOVA with Dunnett's multiple comparison test. ** $P < 0.01$.

induced T cell response days 3 and 8 postinfection (PI). Because the plasma IFN- γ level has previously been shown to peak around day 6 during LCMV infection,²⁷ we also measured cytokines day 6 PI (Figs. 1A, 1B). At day 3 PI, increased levels of plasma IFN- α and IFN- γ were observed. While the IFN- α returned to the basal level day 6 PI, IFN- γ was further increased. This corresponded to a marked CD8 T cell activation, which included the expansion of primed CD8 T cells; these cells have previously been demonstrated to have the capacity to produce large amounts of IFN- γ when triggered by antigen.²⁸ At day 8 PI, plasma IFN- γ level returned to the basal level.

Global Analysis of the RPE/Choroid Transcriptional Response to Systemic Immune Activation From LCMV Infection

To understand the molecular mechanisms in the posterior part of the eye in response to the systemic inflammatory changes, we isolated RPE/choroid days 3 and 8 PI and processed it for whole-transcriptome microarrays. Gene expression levels were compared with uninfected mice and identified as differentially expressed based on a 1.5-fold change criterion and a false discovery rate below 0.1%.

We observed a marked transcriptional response at both time points. At day 3 PI 929 genes were downregulated and 474 genes were upregulated in RPE/choroid of infected mice. The upregulated genes were subjected to functional annotation

using the gene ontology terms. Based on the number of genes implicated, the top 10 list revealed functions relating to inflammation and cell death (Figs. 2A, 2B). At day 8 PI, 176 genes were downregulated and 589 genes were upregulated compared to baseline. As well as pathways associated with inflammation, the functional annotation further included pathways associated with cell activation and adhesion (Figs. 2C, 2D).

Abrogated Interferon Receptor Signaling Markedly Reduces the LCMV Induced Gene Activation in the Retina

Because the LCMV may bind and directly activate retinoic acid inducible gene-1 (RIG-I) and toll-like receptors,^{29,30} of which most are expressed by cultured RPE cells,^{31,32} we sought to determine whether the observed transcriptional changes were due to systemically increased levels of interferons or an off-target effect of the virus. Therefore, we also tested the ocular response to LCMV infection in mice with targeted deficiencies in interferon signaling. The response to type I interferons, which include IFN- α , was tested in mice deficient in the interferon α/β receptor (IFNAR^{-/-}) on day 3 PI, whereas the response to IFN- γ was tested in mice deficient in IFN- γ (IFN γ ^{-/-}) on day 8 PI. Of the genes upregulated in wild type (WT) mice, a large fraction was not upregulated in the mutant strains (Figs. 2A–D). This lack of response was most evident in IFN γ ^{-/-} mice; only about 10% of the genes upregulated at day 8

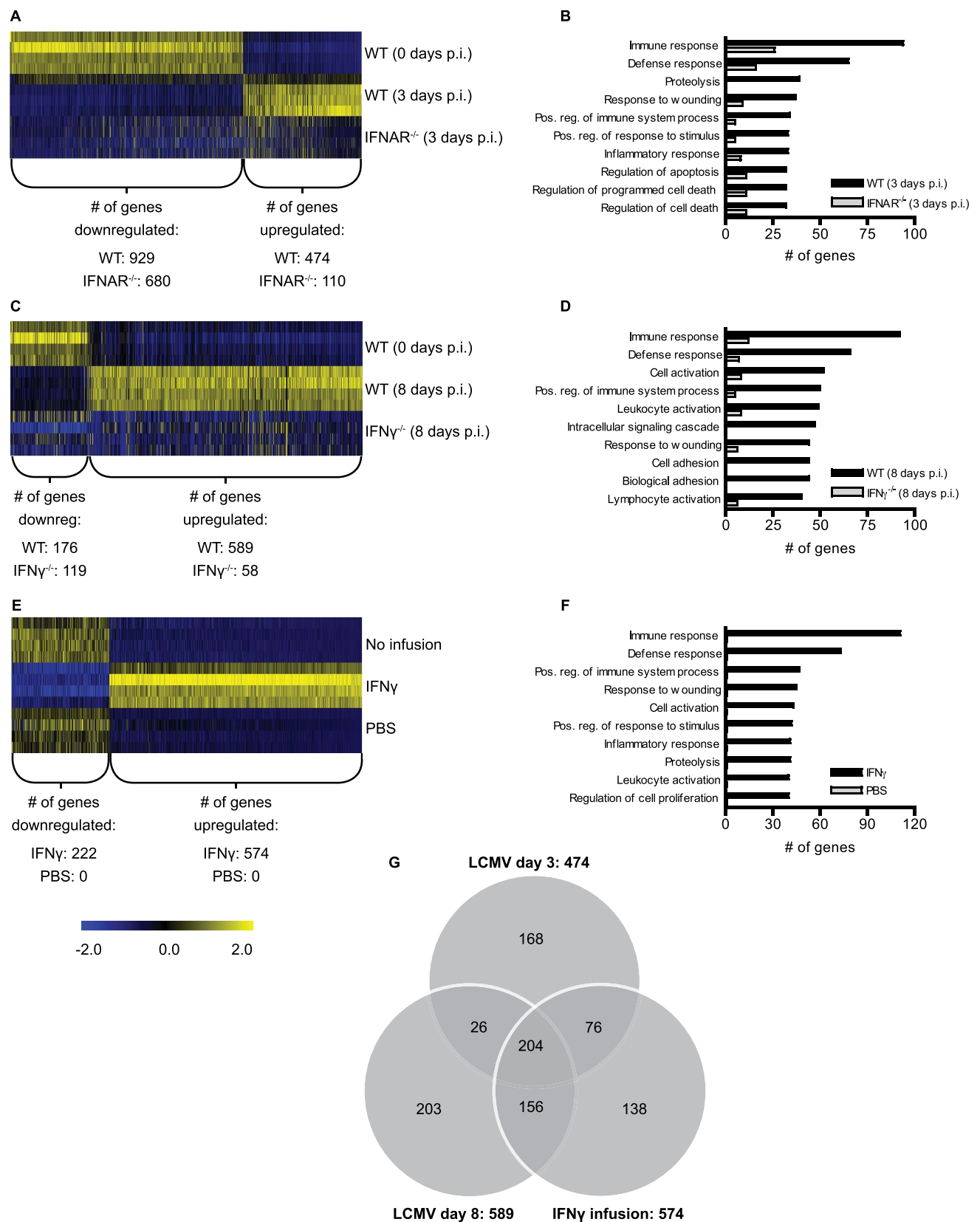


FIGURE 2. Analysis of the RPE/choroid transcriptome from mice with LCMV infection or IFN- γ infusion. (**A**, **B**) Global analysis of differential gene expression in RPE/choroid day 3 PI, (**C**, **D**) day 8 PI and (**E**, **F**) after IFN- γ infusion. The heatmaps display expression patterns of genes significantly up- or downregulated compared to untreated mice according to significance analysis of microarrays using an FDR < 0.001 and a fold change > 1.5. Scale bar represents normalized expression values; yellow denotes upregulation, blue denotes downregulation. (**B**, **D**, **F**) The upregulated genes were annotated according to Gene Ontology terms; shown are the top 10 pathways. (**G**) Venn diagram displaying the number of overlapping genes upregulated in WT mice after infection with LCMV virus or infusion of IFN- γ based on the transcriptome analysis.

PI by LCMV in WT mice were upregulated in $\text{IFN}\gamma^{-/-}$ mice. Notably, this pattern is different from the situation in organs directly supporting viral replication, where absence of $\text{IFN}\gamma$ only moderately modifies the local inflammatory signature.³³

Infusion of $\text{IFN}\gamma$ Mimics the RPE/Choroid Transcriptional Response to Systemic Immune Activation From LCMV Infection

Because we knew from previous experience that even the most sensitive analysis for presence of LCMV cannot exclude minimal virus replication,³⁴ we chose to employ another approach to evaluate the role of circulating $\text{IFN}\gamma$ versus local infection. Thus, we analyzed the RPE/choroid response from the exposure to systemic $\text{IFN}\gamma$ in uninfected WT mice. In a previous study, intraperitoneal bolus injections of $\text{IFN}\gamma$ were not associated with an altered retinal immune status,³⁵ probably because of the short half-life of systemic $\text{IFN}\gamma$.³⁶ Therefore, we infused $\text{IFN}\gamma$ continuously via subcutaneously placed osmotic pumps as previously described.²² At day 5, after start of infusion, we euthanized the mice and isolated ocular tissue and plasma for analysis. The plasma levels of $\text{IFN}\gamma$ in transfused mice averaged 2735 pg/mL (SD: 527) which is comparable to peak levels observed in mice during infection with LCMV²² or murine cytomegalovirus (MCMV).¹⁶ This was significantly increased compared with untreated (mean: 63.8 pg/mL; SD: 23.3) and PBS-infused mice (mean: 44.8 pg/mL; SD: 54.4; Fig. 1D). Similar to LCMV infection, systemic $\text{IFN}\gamma$ exposure also had a marked effect on the RPE/choroid transcriptome—inducing the upregulation of 574 genes. No genes were differentially regulated in the PBS-infused mice, thus confirming that the observed gene regulation in $\text{IFN}\gamma$ -infused mice was not secondary to the surgical procedure or any irritant effects from the pumps, but represented a response specific to the $\text{IFN}\gamma$ exposure (Fig. 2E). A great overlap was observed in the top 10 gene ontology terms inferred from the functional annotation of the upregulated genes compared with the functional annotation of LCMV-infected mice (Fig. 2F). Consistently, 76.0% of the $\text{IFN}\gamma$ -induced genes were also upregulated in RPE/choroid of LCMV-infected mice day 3 or 8 PI (Fig. 2G).

Systemic Immune Activation From LCMV Infection Induces Upregulation of Chemokine Ligands in RPE/Choroid Through Interferon Signaling

Systemic immune activation from LCMV infection-induced RPE/choroid expression of several chemokine-encoding genes, encoding chemoattractants known to be involved in recruitment of inflammatory cells. Because of their probable influence on the immune state of the chorioretinal interface, we analyzed this family of chemoattractants separately (Fig. 3). The transcripts *Ccl2*, *Ccl3*, *Ccl5*, *Ccl6*, *Ccl7*, *Ccl8*, *Cxcl9*, *Cxcl10*, *Cxcl11*, *Cxcl12*, and *Cxcl16* were all upregulated day 3 and/or day 8 PI. While few of these chemokines were upregulated in the interferon mutant mice strains, the majority were upregulated after $\text{IFN}\gamma$ infusion. Additionally, of chemokines not induced by LCMV infection, the $\text{IFN}\gamma$ infusion also induced the upregulation of *Xcl1* and *Ccl19* (Fig. 3C). Together, this demonstrates that interferons are essential for the LCMV-induced upregulation of chemokines in RPE/choroid, and that systemically derived $\text{IFN}\gamma$ is sufficient to induce such upregulation in vivo.

Although previous results obtained from gene expression microarrays have been shown to correlate strongly with RT-PCR^{17,19} and protein^{13,14,17} findings of the RPE and RPE/choroid, we proceeded to measure the protein levels of

selected chemokines in lysates of RPE/choroid from LCMV-infected and $\text{IFN}\gamma$ -infused mice (Fig. 4A). Consistent with the transcriptomics data, increased levels of CCL2 and CCL3 were found in LCMV-infected mice. Corresponding to the relatively high gene expression levels detected, increased levels of CCL5 and CXCL9 were found in both LCMV-infected and $\text{IFN}\gamma$ -infused mice. To examine if the increased local levels of chemokines were simply spillover from the blood present in the choroid tissue lysates, we also measured protein concentrations of these chemokines in blood plasma. We found increased plasma levels of CCL5 and CXCL9 in LCMV-infected mice and increased plasma level of CXCL9 in $\text{IFN}\gamma$ -infused mice (Fig. 4B). When related to total protein content, the level of CCL3 was higher in RPE/choroid than in plasma in LCMV-infected mice, and the level of CCL5 was higher in RPE/choroid than in plasma in both LCMV-infected mice and $\text{IFN}\gamma$ -infused mice. Together, this indicates that the measured protein reflects local synthesis, and therefore that the observed gene upregulation translates to increased protein levels of these analytes.

Differential Gene Expression Suggests RPE/Choroid Recruitment of Leukocytes During Systemic Immune Activation From LCMV Infection

The induced chemokine ligands are known to attract several types of leukocytes including monocytic lineage cells (monocytes, macrophages, microglia, and immature dendritic cells) granulocytes (neutrophils, basophils, and eosinophils) and lymphocytes (Type 1 and 2 helper, cytotoxic effectors, memory T cells, B and NK cells).^{37,38} Consistent with this, the transcripts *Ccr2*, *Ccr5*, *Cxcr3*, *Cxcr6*, and *Cx3cr1* were upregulated day 8 after infection (Fig. 5). These encode chemokine receptors present on several types of leukocytes including microglia/macrophages and antigen-primed T cells,^{37,38} suggesting a chemokine-dependent recruitment of T cells and microglia/macrophages to the RPE/choroid. Consistent with this, the transcriptomic change day 8 PI included both upregulation of several genes implicated in cellular adhesion (*Icam1*, *Vcam1*, and *Thy1*) and the interacting integrins (*Itgb2* [encoding CD18], *Itgal* [CD11a], *Itgam* [CD11b]). Further, several transcripts expressed by microglia/macrophages (including *Cd36*, *Cd45*, *Emr1* [F4/80] and *Cd86*) and T cells (including *Cd2*, *Cd3e*, *Cd3g*, *Cd8a*, *Gzma*, and *Gzmb* [encoding granzymes A and B]) were also upregulated (data not shown).

Systemic Immune Activation From LCMV Infection Induces Upregulation of Early Complement Genes in RPE/Choroid Through Interferon Signaling

It has been suggested that the RPE/choroid contains a local complement system with functional implications for retinal immune homeostasis and pathology.^{39,40} Therefore, we analyzed the group of complement-encoding genes separately. We found that all transcripts encoding the subunits necessary for assembly of the C1 molecule and the classical C3 convertase were upregulated after systemic infection with LCMV. The concomitant upregulation of *C3* and *Cfb*, encoding the substrate for the classical C3 convertase and all subunits necessary for the formation of the alternative C3 convertase, may enable classical complement activation and amplification of the C3 cleavage through the alternative amplification loop. We also noted upregulation of C4, recently invoked as a T-cell intrinsic regulator of inflammation in experimental autoimmune uveitis.⁴¹ Notably, of the complement-encoding genes upregulated in WT mice in response to LCMV, only *Cfb* was

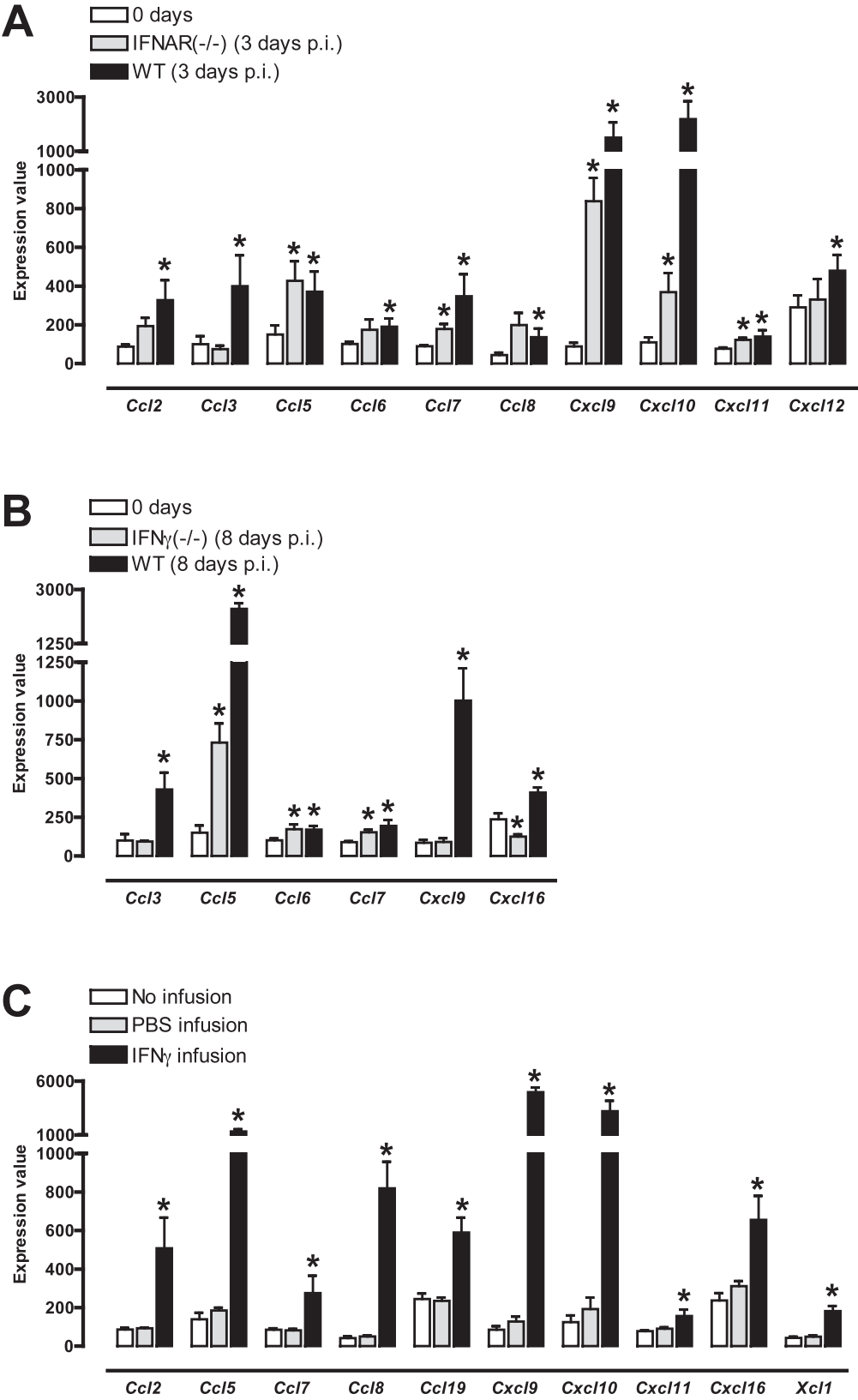


FIGURE 3. Chemokine-ligand-encoding transcripts upregulated in the RPE/choroid after (A, B) LCMV infection or (C) IFN- γ infusion. Shown are mean expression values of genes significantly upregulated compared to untreated mice, according to significance analysis of microarrays using an FDR < 0.001 and a fold change > 1.5. Asterisks denote significance according to this method. Error bars represent standard deviation.

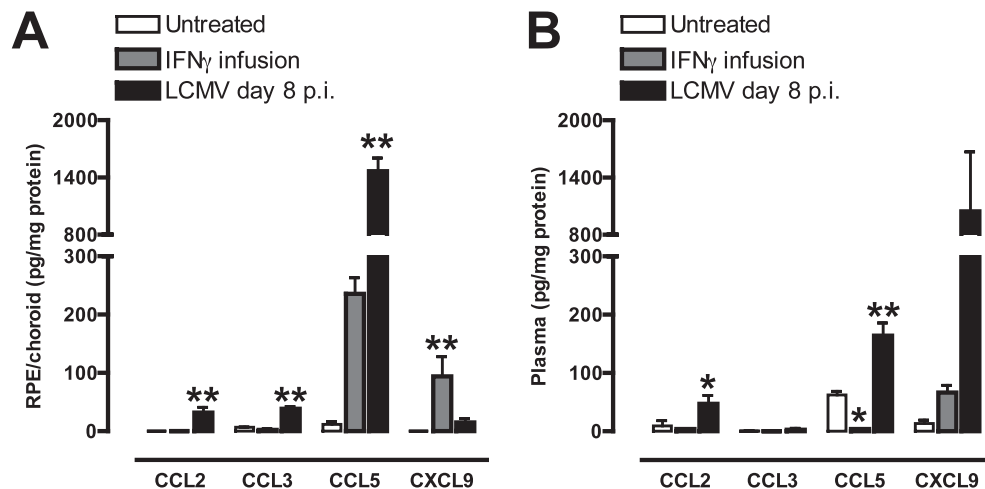


FIGURE 4. Protein concentration of chemokines in (A) lysates of RPE/choroid and (B) plasma after LCMV infection or IFN- γ infusion. Protein concentrations were normalized to protein content as measured in the BCA assay. Asterisks denote significance compared with untreated mice; 1-way ANOVA with Dunnett's multiple comparison test. * $P < 0.05$; ** $P < 0.01$.

upregulated in the interferon mutant mice strains, and all were upregulated in WT mice following IFN- γ infusion. This demonstrates a marked dependence on the interferons in upregulation of complement genes in the RPE/choroid following systemic immune activation from LCMV infection and that systemically derived IFN- γ is sufficient to induce such upregulation in vivo (Supplementary Fig. S1).

DISCUSSION

To characterize interactions between the RPE/choroid and the immune system in vivo, we measured transcriptional changes in RPE/choroid in response to systemic activation of the immune system. Not only T cell activation from infection with intravenous LCMV virus, but also the continuous infusion of recombinant IFN- γ resulted in upregulation of genes associated with inflammation in the RPE/choroid.

The overlap of upregulated genes between the IFN- γ -infused uninfected mice and LCMV-infected WT mice demonstrate that the induction of chemokine genes—to a large extent—represents a specific response to the systemic immune activation and cytokine release triggered by the

infection, and not just an off-target effect of the LCMV infection. This conclusion is also supported by the fact that there are distinct differences in the kinetics, but not identity, of cytokine and chemokine genes expressed in the RPE/choroid, compared with what has previously been described in virus-infected organs such as the LCMV-infected central nervous system.⁴² In directly virus-infected tissue, the IFN-induced inflammatory profile is maintained and substantially augmented following the recruitment of virus-specific effector T cells and subsequent local antigen recognition.⁴³ In the RPE/choroid, this secondary augmentation was absent. Plasma levels of IFN- α and IFN- γ returned to basal levels at day 6 and 8 PI, respectively (Fig. 1B), and notably, the RPE/choroid gene expression levels of these cytokines were low in unstimulated mice and did not increase at day 3 or 8 PI (data not shown). Thus, a high level of circulating IFN- γ appears to be intrinsically capable of inducing the upregulation of chemokine genes in the RPE/choroid.

Inflammatory stimulation has previously been shown to induce a range of chemokines in RPE cells. Of the chemokines upregulated in RPE/choroid of virus-infected and IFN- γ -infused mice, *CCL2*, *CCL5*, *CCL7*, *CCL8*, *CXCL9*, *CXCL10*, and *CXCL11* have been shown to be induced in human RPE in vitro by

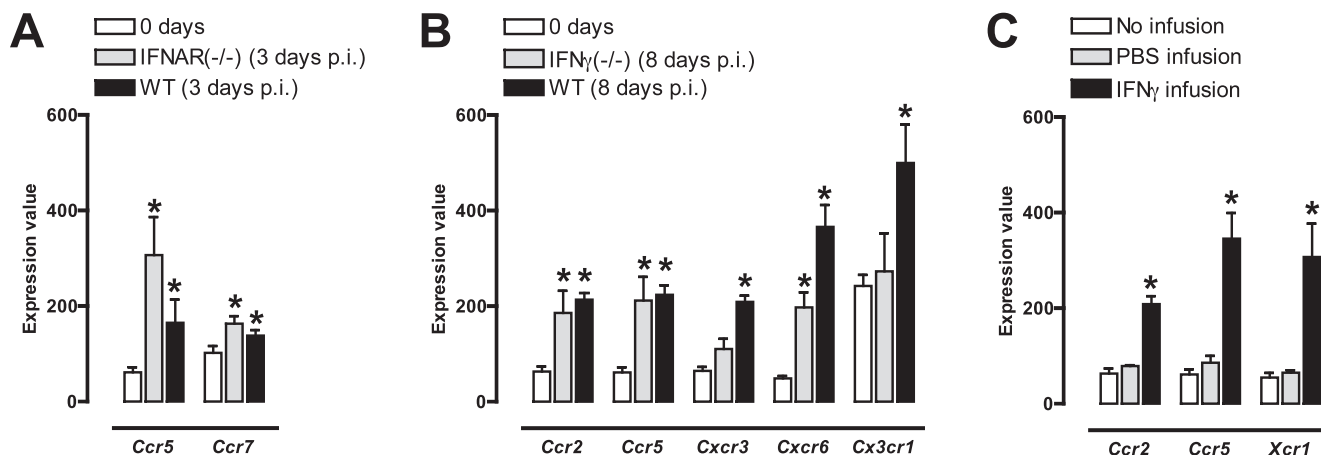


FIGURE 5. Chemokine-receptor-encoding transcripts upregulated in the RPE/choroid after (A, B) LCMV infection or (C) IFN- γ infusion. Shown are mean expression values of genes significantly upregulated compared to untreated mice, according to significance analysis of microarrays using an FDR < 0.001 and a fold change > 1.5 . Asterisks denote significance according to this method. Error bars represent standard deviation.

soluble mediators from activated T cells or the combination of TNF- α and IFN- γ .^{13,44} In line with this, increased RPE/choroid expression of *Ccl2*, *Ccl5*, *Ccl8*, *Cxcl9*, *Cxcl10*, and *Cxcl16* have been observed in aged mice^{17,45} and induction of *Ccl2* has been demonstrated in mouse RPE/choroid in response to laser-induced choroidal neovascularization.⁴⁶ In agreement with our results, in vivo studies have also reported ocular accumulation of leukocytes in response to the increased levels of chemokines at the chorioretinal interface^{17,45–47} or in response to neuroretinal IFN- γ expression.⁴⁸ Furthermore, a systemically increased level of IFN- γ was shown to induce the activation and relocation of retinal CD45⁺ Iba1⁺ F4/80⁺ CD86⁺ microglia to the subretinal space.¹⁶ Notably, in our system, transcripts for all these macrophage/microglia markers were significantly increased in the RPE/choroid by systemic LCMV infection as well as IFN- γ infusion. Finally, the findings of increased transcripts of *CXCL9* and *CXCL10*⁴⁹ and CXCL10 protein⁵⁰ in RPE/choroid from patients with AMD and increased levels of several chemokines including CCL2, CXCL9, and CXCL10 in vitreous fluid from patients with neovascular AMD⁵¹ and diabetic retinopathy^{52,53} corroborate the significance of chemokines to retinal pathology.

The diversity of stimuli that induce the upregulation of chemokine- and complement-encoding genes in RPE/choroid suggests that this response represents a final common pathway of the RPE/choroid. It is undetermined whether this response is beneficial or detrimental. However, given that it can be induced by stimuli such as systemic infection with a low-virulence virus or short-term exposure to IFN- γ , stimuli that may be encountered multiple times during a lifetime, this hardly represents a detrimental response on its own. Rather, it may serve beneficial functions essential to tissue homeostasis in the setting of acute systemic inflammation. Thus, the C1 molecule may serve to dampen inflammation locally through binding to and facilitating the removal of apoptotic cells and debris by macrophages or retinal microglia that are attracted by the increased levels of chemokines.^{38,54,55} However, in a state of chronic inflammation, the outcome may be detrimental. Thus, mice chronically and systemically infected with murine cytomegalovirus developed a more pathologic response to laser-induced choroidal neovascularization.⁵⁶ Also, the retinal degeneration observed in aged mice of the A/J genetic background has been explained by an augmented interferon signaling already at a young age prior to development of the pathologic changes of the retina.⁵⁷ Together these observations indicate that chronic, systemic inflammation may contribute to the development of retinal pathology.

Strengths of this study include the in vivo approach for measuring the RPE/choroid response to systemic immune stimulation, and the use of whole transcriptome microarrays, allowing unbiased examination of transcriptional responses. Limitations include the short-term stimulation and lack of functional assays. Thus, further studies are needed to determine the functional effects of the RPE/choroid response to acute immune activation and to determine whether such effects are altered by the duration of stimulus, or other factors, such as age. Further insights into this response system may prove useful in designing therapeutic strategies aimed at inhibiting or promoting the function of immune cells at the RPE/choroidal interface.

In conclusion, with the purpose of characterizing the interactions between the RPE/choroid and the immune system in vivo, we analyzed the RPE/choroid transcriptional response to acute systemic activation of the immune system. This response included a marked upregulation of early complement components and chemokine ligands and receptors. While these findings were largely absent from mice deficient in IFN- γ , the continuous systemic infusion of IFN- γ sufficed to induce

the upregulation of most of the chemokines induced by systemic virus infection. Together, these findings indicate that acute systemic immune activation, probably through increased levels of proinflammatory mediators in the circulation, triggers a local response at the RPE/choroid interface that may include chemokine-dependent recruitment of inflammatory cells and engagement of the complement system. If this also is the case after systemic, chronic, low-grade inflammation, this may represent a link between the observed systemic inflammatory changes and the retinal pathology in several multifactorial entities such as aging, AMD, and diabetes.

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